Analysis of rumen bacterial populations in dairy cattle fed different forages.

Undergraduate Research Thesis

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Abstract

Forage is an important component of diets for ruminant animals. Rumen bacteria are responsible for degrading fiber in forages. In addition, microbial proteins make up the major portion of the proteins that the animal digests and absorbs in the small intestines. Different species of bacteria are better suited to increase efficiency of rumen degradation than others. Also, methane production by methanogens in the rumen results in a 6 to 10% loss of energy in feed; this is an increasingly pressing issue for both the health of the environment and the efficiency of the producer. Changes in types and amounts of forages in the diet can alter the populations of the various bacterial species in the rumen, the rumen fermentation, as well as populations of methanogens. The objective of this study was to determine whether changes in the types and amount of forage change the populations of methanogens and fibrolytic bacteria species. In the present study, five rumen-cannulated Holstein cows were each fed five diets with different forage sources over a period of fifteen weeks in a Latin Square design, with each diet being fed for three weeks to each cow. The five diets were: corn silage as a control diet, the control diet plus 5.1% wheat straw, 10.1% wheat straw, 5.5 % corn stover, or 11.5% alfalfa hay. The rumen samples collected were filtered through three layers of cheesecloth to separate the liquid and solid fractions, and microbial DNA was extracted from each fraction of each sample. Specific quantitative real-time PCR was used to analyze the populations of three common rumen cellulolytic bacterial species: *Ruminococcus albus, Fibrobacter succinogenes,* and *Ruminococcus flavefaciens*. In addition, three novel species from the genera *Acetivibrio, Acidaminobacter,* and *Lachnospira,* which were identified from fractionated rumen samples in a previous study, were assessed as well. Populations of total bacteria, the genus *Butyrivibrio,* and methanogens (archaea) were also analyzed. DGGE of total bacteria and archaea was performed

to assess the dietary effects on the overall bacterial and archaeal communities. No differences were observed between the five diets, indicating that the differences between the forages offered was not sufficient to alter the bacterial and archaeal populations.

Introduction

Forage is an important component of diets for ruminant animals, and is vital in maintaining rumen function and health. Rumen bacteria play a major role in degrading fiber in forages, allowing the animals to digest the diet and to make use of the energy present in cellulose, hemicellulose, and other fibrous components in the forage. In addition, proteins that make up the ruminal bacteria themselves constitute the major portion of the proteins that the animal digests and absorbs. Without bacterial proteins, ruminants would not be able to meet all of their amino acid requirements, making it nearly impossible for dairy production to be efficient and profitable. Therefore, it is important that a diet contains enough degradable forage to maintain a bacterial population and allow for maximal productivity from dairy cattle.

Rumen bacteria must attach to feed particles in order for degradation to take place. Multiple species form a biofilm on the surface of the particles, with the species present depending on the type of substrate (McAllister et al., 1994). Thus, changes in a diet can alter the populations of the various bacterial species in the rumen. For example, increasing forage levels in the diet can increase the concentration of cellulolytic species, while increasing concentrate levels will increase the number of starch-degrading bacteria. Larue et al. (2005) found that sheep fed an all grass hay diet had large amounts of *Clostridia* present in the rumen microbiota, while those fed a starch diet had greater amounts of *Selenomonas* and *Ruminococcus* species. In addition, certain species may be better suited to degrade certain components of forage. For

example, *Fibrobacter succinogenes* has been shown to be more efficient than *Ruminococcus albus* at degrading hemicellulose (Matulova et al., 2008). *Butyrivibrio fibrisolvens* has been shown to degrade hemicellulose better than cellulose, while *Ruminococcus flavefaciens* can degrade either fiber (Miron, 1994). Forage sources can also affect bacterial activity: Dehority and Scott (1967) showed that *Bacteroides ruminicola* (now *Prevotella ruminicola*) can degrade hemicellulose from alfalfa more efficiently than that of bromegrass, and Miron (1991) found that *Fibrobacter succinogenes* could degrade alfalfa more efficiently than many other fibrolytic species. Feeding forages that are easier for fibrolytic bacteria to degrade and that encourage colonization by certain bacterial species could potentially increase the amount of fiber utilized, alter feed efficiency or enhance functional characteristics of milk and other animal products. For example, research has suggested that high moisture corn could decrease milk fat concentration when compared to feeding dry ground corn (Bradford and Allen, 2004).

Forage type could also affect the amount of methanogens in the rumen. Methanogens can use some of the rumen fermentation products to produce methane during the ruminal fermentation processes. Methane contains energy, but ruminants cannot use it, resulting in a loss of energy to the environment. According to Nicholson et al. (2006), between 6 and 10% of the energy present in the digested feed is lost via methane production. Losses due to methane production result in a decreased feed and production efficiency and increase the overall production costs for producers. More importantly, methane is also a potent greenhouse gas that may contribute to global warming. Feeding forages that reduce methanogen production could be more efficiently utilized by the animal, decreasing the amount of feed needed to fulfill their energy requirements. Methanogen species present in the rumen appear to be similar across ruminant species (Nicholson et al., 2006), indicating that information about forage utilization

could be applied in numerous species and production settings in order to improve efficiency and reduce environmental impact.

The objective of this study was to investigate whether different types of forages have an effect on fibrolytic species in the rumen, and to what extent these effects may be. The research examined how individual bacterial populations respond to the amount and type of forage being fed. Methanogen populations were also examined to determine whether there is a relationship between types and amounts of forages and the number of methanogens present.

Materials and Methods

Feeding Trial

Five rumen-cannulated, lactating Holstein cows were fed a series of five different diets over 15 weeks, with each diet being fed for a period of 3 weeks. The diets were formulated to have 17.9% crude protein and 38% non-fiber carbohydrates. All diets contained 18% forage neutral detergent fiber (NDF), with exception of the 10.1% wheat straw diet, which had 22% NDF (Table 1).

This study was approved under Animal Use Protocol #05-AG018 and followed established practices for humane care of animals.

Table 1. Dietary treatments utilized to assess rumen bacterial population species and concentration [a].

Added Forage	Corn Silage	Grain Mix
None (Control)	44.10%	55.90%
11.5% Alfalfa Hay	34.00%	54.50%
5.1% Wheat Straw	34.00%	60.90%
5.5% Corn Stover	34.00%	60.50%
10.1% Wheat Straw	36.10%	53.80%

[a]Animals were provided *ad libitum* access to the diets and were not given access to pasture.

Rumen Sample Collection

Rumen digesta samples were collected from each cow during the 3rd week of each feeding period on one of the five diets (for a total of five samples per diet), allowing 2 weeks for the rumen bacteria to adjust to the new diet. A rumen sample was also collected prior to the initiation of the feeding trial to serve as a baseline for comparison with the treatment samples. Samples were collected 6 hours after feeding. The samples were separated into solid and liquid fractions by squeezing through three layers of cheesecloth. The liquid fraction of the sample was centrifuged at 350g for 15 minutes. One mL of the supernatant was transferred to a 2-mL microcentrifuge tube and centrifuged again at 10,000g for 20 minutes at room temperature to harvest the bacterial biomass. Microbial DNA was then extracted from the pelleted bacteria. The solid fraction was washed in PBS buffer and shaken gently for 30 seconds, then centrifuged at 500g for 15 minutes. The supernatant was removed and the pelleted plant particle fraction was transferred to a 2-mL microcentrifuge tube for microbial DNA extraction.

DNA Extraction

DNA was extracted using the RBB+C method as described by Yu and Morrison (2004). The fractions of each sample were added to a separate 2mL screw-cap tube along with 0.4g of sterile zirconia beads (0.3g of 0.1mm beads and 0.1g of 0.5mm beads). For the solid fractions, 0.5g were added to the tubes. One mL of lysis buffer was added to each tube, and the samples were homogenized in a Mini-Beadbeater for 3 minutes to break up the cell wall and membranes. The samples were incubated at 70ºC for 15 minutes, then centrifuged at 16,000g for 10 minutes at 4 ºC. The supernatant was transferred to a fresh 2-mL microcentrifuge tube. To increase the

efficiency of the extraction, 300µl of lysis buffer was added to the screw-cap tube and the tube was again homogenized, incubated and centrifuged, with the fresh supernatant being combined with the previous supernatant that was collected.

To precipitate the impurities, ammonium acetate was added to each tube and the samples were incubated on ice for 5 minutes, followed by centrifugation for 10 minutes at 16,000g and 4 ºC. The supernatant was transferred to a fresh 1.5mL microcentrifuge tube. Then an equal volume of isopropanol was added, and the samples were incubated on ice for 30 minutes. After centrifuging the tubes for 15 minutes, the supernatant was aspirated and the nucleic acid pellet was washed in 70% ethanol, dried and then dissolved in Tris-EDTA buffer.

To remove RNA from the samples, RNase was added and the tubes were incubated at 37ºC for 15 minutes. Proteinase K was added and the samples were incubated at 70ºC for 10 minutes to remove any proteins present. The QIAamp DNA Mini Kit was used to purify the DNA samples. Once extraction was complete, DNA quality was checked by agarose gel (1.5%) electrophoresis.

Real-Time PCR

Real-Time PCR was used to assess total bacteria; archaea; the genus *Butyrivibrio*; three commonly cultured fibrolytic species: *Fibrobacter succinogenes, Ruminococcus albus,* and *Ruminococcus flavefaciens;* and three novel rumen species: *Acetivibria* sp. Ad-H1-14, *Acidaminobacter* sp. Ad-H2-90, *and Lachnospira* sp. Ad-C2-50. A standard for each group was created using PCR product from a pool of the samples. The basic thermoprofile used for the production of the standard was 94º/5’; 35x(94º/30”, [primer annealing temperature]/30”, 72º/2’); 72º/10’; 4º/0; End (Table 2).

Table 2. Primers used in the analysis of ruminal bacteria populations across dietary treatments.

Group Analyzed	Primer	Annealing Temperature
Total Bacteria	63f/1389r	55ºC
Archaea	787f/1059r	60ºC
Butyrivibrio	Univ-530f/Buty-900r	65ºC
Ruminococcus albus	Ra1281f/Ra1439r	55ºC
Ruminococcus flavefaciens	Rf154f/Rf425r	55ºC
Fibrobacter succinogenes	Fs-F/Fs-R	55ºC
Acetivibria Ad-H1-14	Univ-530f/Ad-H1-14r	54ºC
Acidaminobacter Ad-H2-90	Univ-530f/Ad-H2-90r	54ºC
Lachnospira Ad-C2-50	Univ-530f/Ad-C2-50r	54ºC

The PCR product was confirmed using agarose gel (1.5%) electrophoresis. The product was then quantified using the Quant-iT dsDNA Assay Kit. Total DNA in the individual samples was also quantified this way for use in later statistical analysis. Serial dilutions were made of each standard for use in real-time PCR.

Primers used to make the standard were also used for real-time PCR, with the exception of total bacteria, whose abundance was quantified using the 340f/806r primers with a TaqMan probe at an annealing temperature of 60 ºC (Nadkarni et al., 2002).

DGGE

PCR and Denaturant Gradient Gel Electrophoresis (DGGE) were used to analyze total bacterial and archaeal populations using the Ingeny PhorU-2 system. Primers pairs GC-ARC344f/EUB519r and GC-EUB357f/EUB519r were used to amplify the PCR product for archaea and bacteria, respectively. The following thermoprofile was used: 94º/4’; 10x(94º/30”, 61º/30”, -0.5º/cyc, 72º/30”); 25x(94º/30”, 56º/30”, 72º/30”); 72º/30’; 4º/0; End. PCR products were tested for quality using agarose gel (1.5%) electrophoresis. DGGE was performed for

archaea using a 40%-60% denaturant gradient (100% gradient was defined with 40% formamide and 7M urea). For total bacteria, the denaturant gradient was reduced to 40%-55% in order to increase resolution of the bands. The archaea DGGE was run for 1 hour at 80V (to pull the product through the stacking gel) before switching to 160V for 17 hours. The total bacteria DGGE was run for 1 hour at 80V, 15 hours at 160V. 10µl of PCR product was loaded into each lane on the DGGE gels. Gels were stained using SYBR green I at a ratio of 1µl SYBR to 10ml of buffer solution, and rocked gently for 30 minutes. Gel images were obtained using a FlourChem 8000 imager.

Statistical Analysis

Statistical analysis of the real-time PCR data was performed using SAS 9.1. Treatment effects were analyzed to assess relationships among populations. Cluster analysis from the DGGE gel images was performed using Bionumerics 5.10. Principle component analysis was performed on the Bionumerics output data using the PCORD5 computer program.

Results

Quantitative RT-PCR

There was no difference in bacterial population sizes between any of the diets with regard to the five treatments ($P > 0.05$). Statistical analysis did indicate that some groups had period effects: *Acetevibria* sp. Ad-H1-14, *Acidaminobacter* sp. Ad-H2-90, *Butyrivibrio*, and total bacteria from the liquid fraction; and *Butyrivibrio, F. succinogenes, archaea*, and *Lachnospira* sp. Ad-C2-50 from the solid fraction each had period p-values less than 0.05.

Across treatments, the average number of bacterial copies in each dietary treatment group tended to be larger in the solid fraction than the liquid fraction (Figure 1). The average value for total bacteria was $5.21x10^5$ and $8.96x10^5$ copies/ng of total DNA extracted in the liquid fraction and the solid fraction, respectively (Figure 2). Archaea values averaged $9.47x10^1$ copies/ng in the liquid fraction and $1.15x10^2$ in the solid fraction (Figure 3). *Butyrivibrio, R. albus, R. flavefaciens,* and *Acetevibria* sp. Ad-H1-14 all had populations on the scale of 10^3 in both fractions (Figure 4-6, 8); *F. succinogenes* had copies on the scale of 10^2 in both fractions (Figure 7), and *Acidaminobacter* sp. Ad-H2-90 and *Lachnospira* sp. Ad-C2-50 both had populations on the scale of 10^2 in the liquid fractions and 10^3 in the solid fractions (Figures 9 & 10). Population values varied greatly between treatments, as shown by the standard deviations for each treatment.

Standard deviations within treatments showed some differences between solid and liquid fractions of the samples. Differences were observed for *Ruminococcus flavefaciens* and *Acidaminobacter* sp. Ad-H2-90 in the corn silage, 10.1% wheat straw, and alfalfa diets. *Butyrivibrio* exhibited differences in all diets with the exception of alfalfa (Figures 4, 6 & 9).

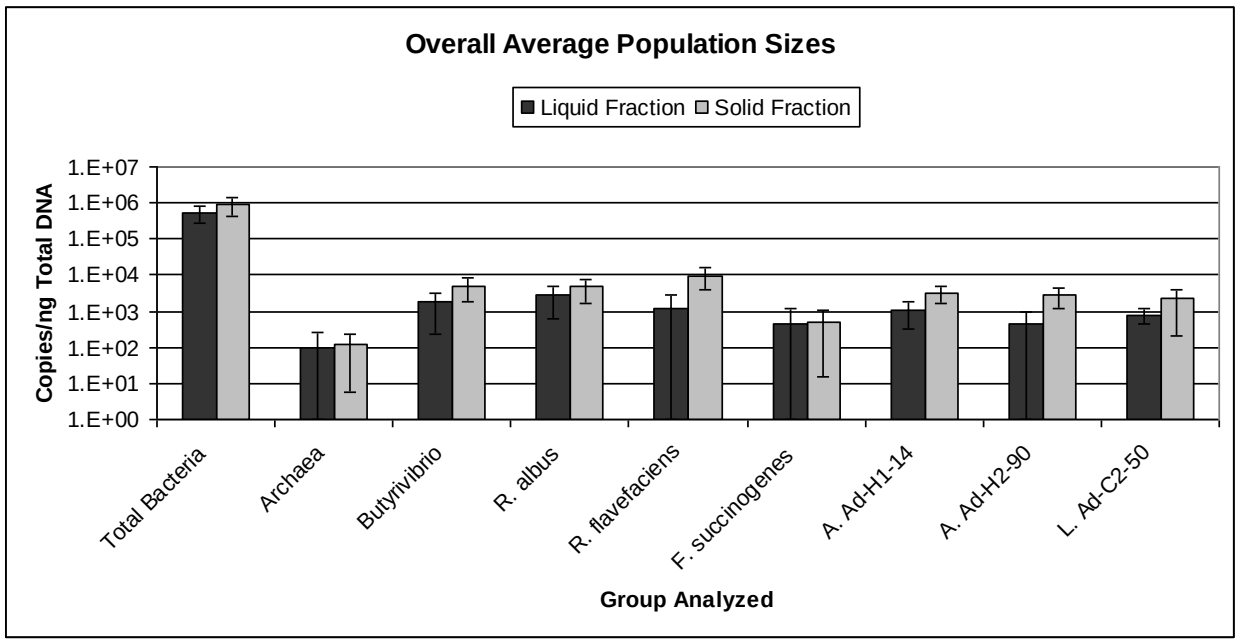

Figure 1. Bacterial and archaeal population sizes across dietary treatments for the liquid and solid fractions. Note: standard deviations were calculated across treatments.

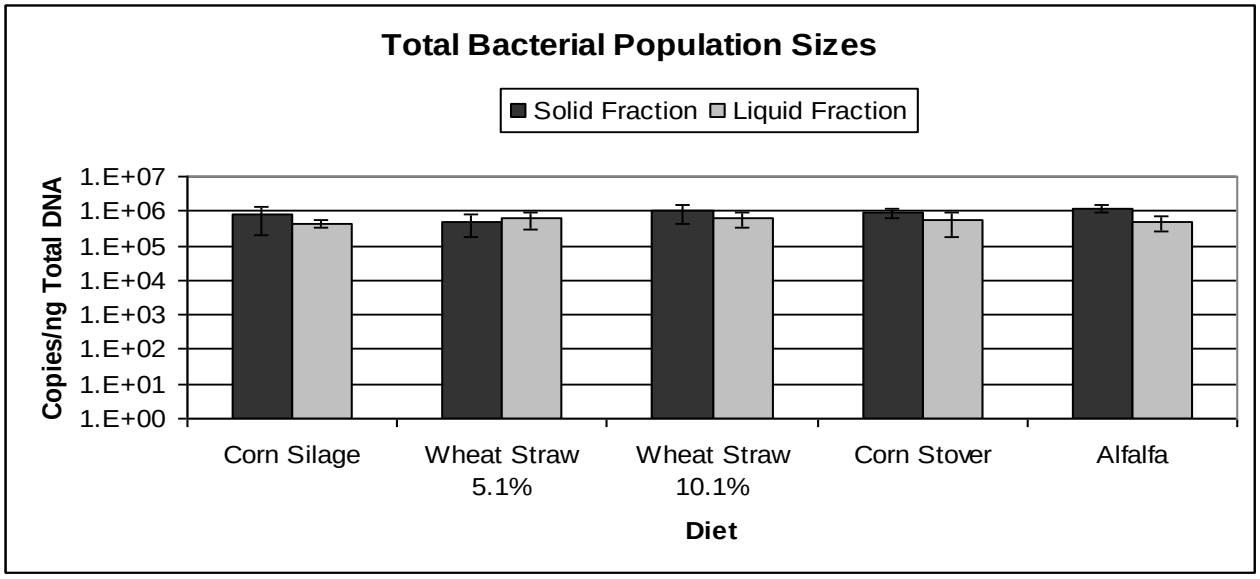

Figure 2. Total bacterial population sizes within the solid and liquid fractions for each dietary treatment.

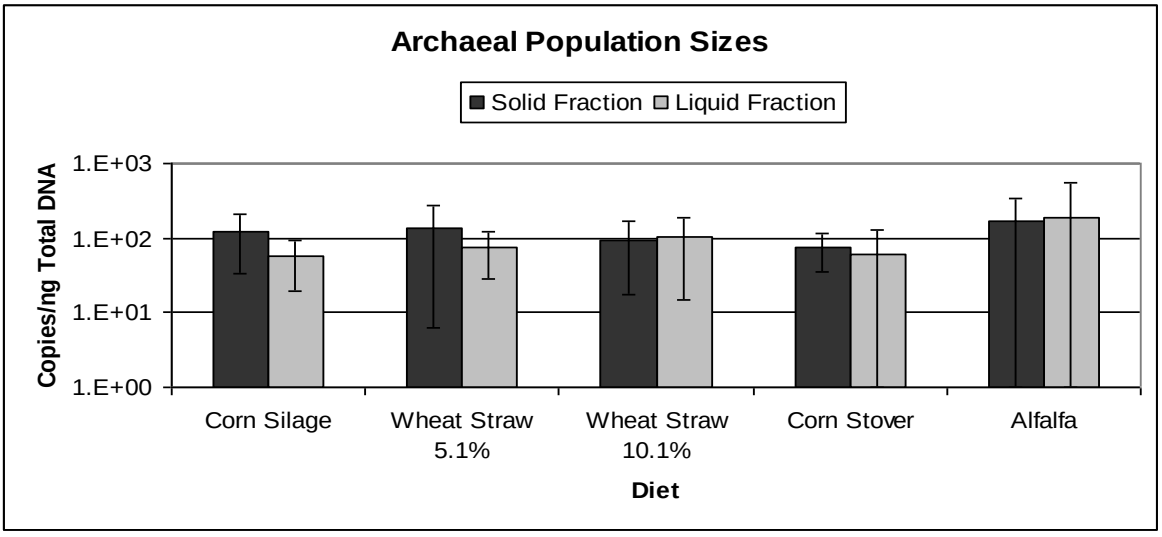

Figure 3. Archaeal population sizes within the solid and liquid fractions for each dietary treatment.

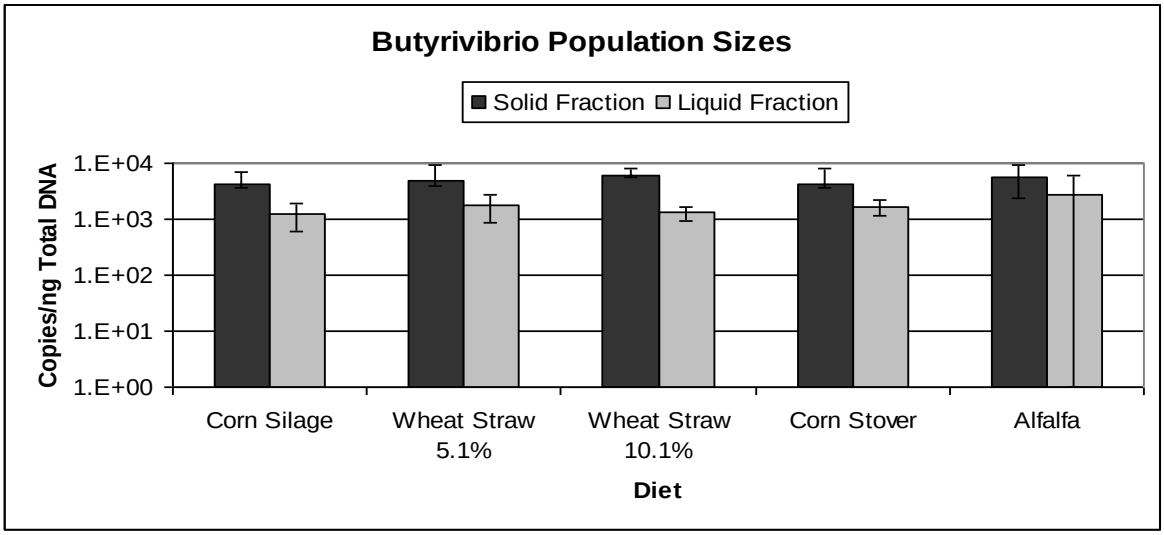

Figure 4. Population size of *Butyrivibrio* within the solid and liquid fractions for each dietary treatment.

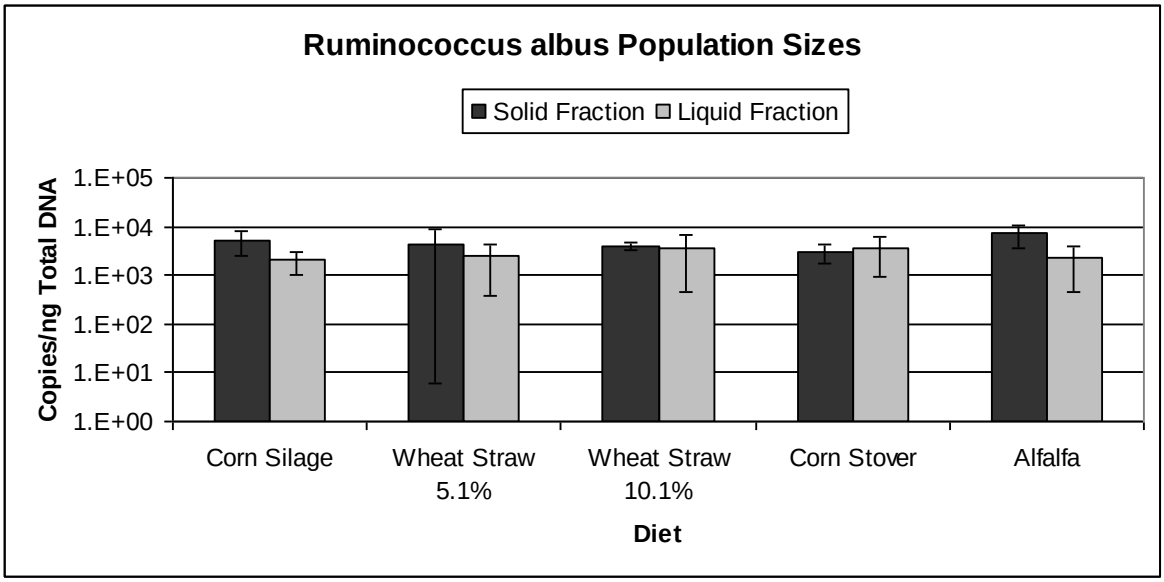

Figure 5. Population size of *Ruminococcus albus* within the solid and liquid fractions for each dietary treatment.

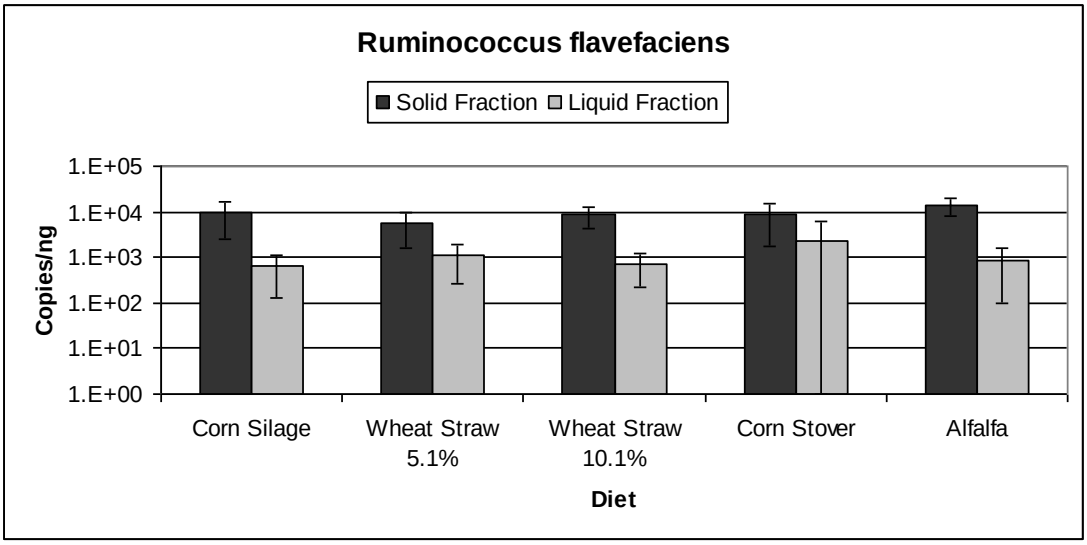

Figure 6. Population size of *R. flavefaciens* within the solid and liquid fractions for each dietary treatment.

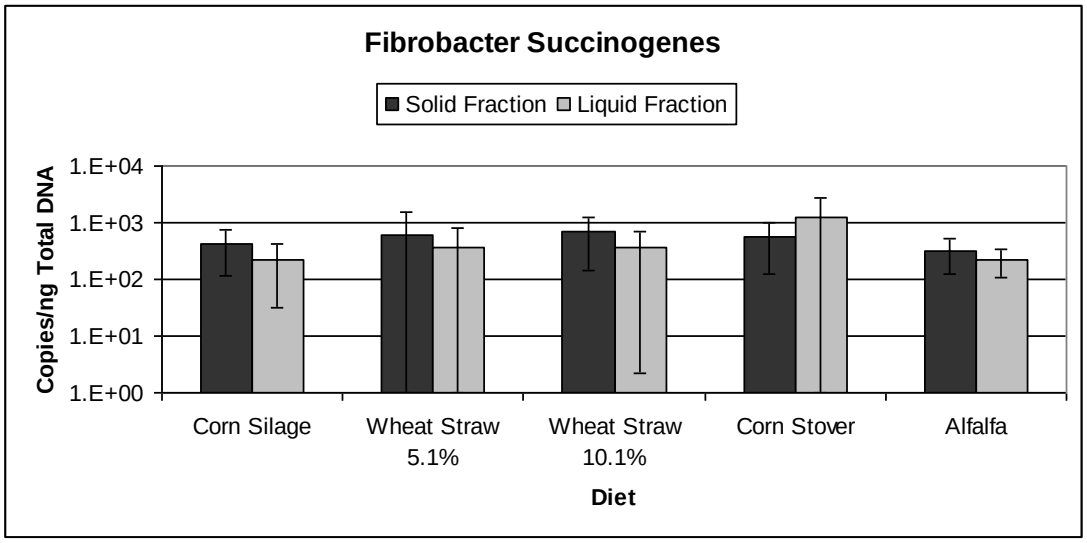

Figure 7. Population size of *Fibrobacter succinogenes* within the solid and liquid fractions for each dietary treatment.

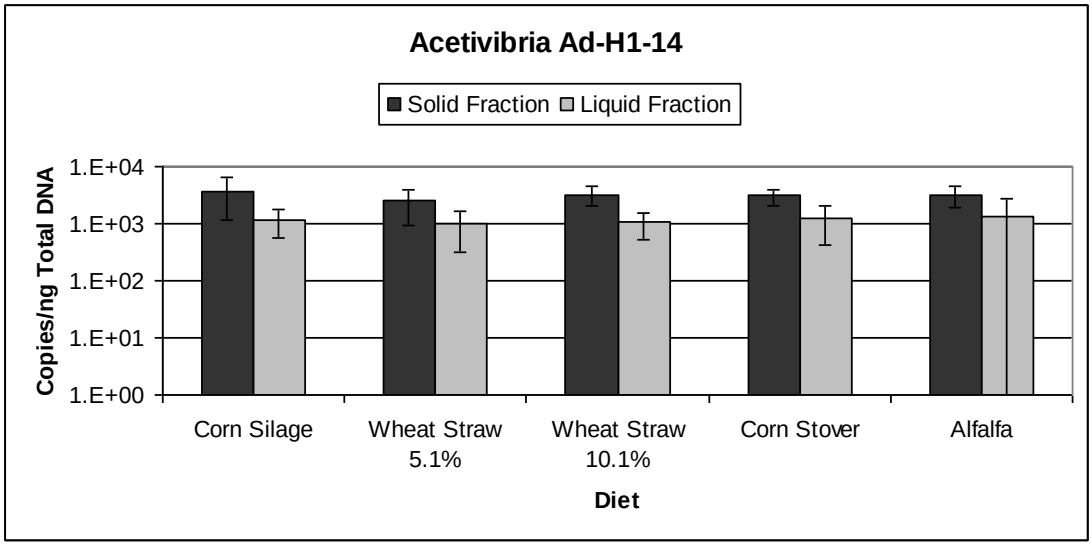

Figure 8. Populations of *Acetivibria* sp. Ad-H1-14, and uncultured bacterium recovered from the adhering fraction of rumen content, within the solid and liquid fractions for each dietary treatment.

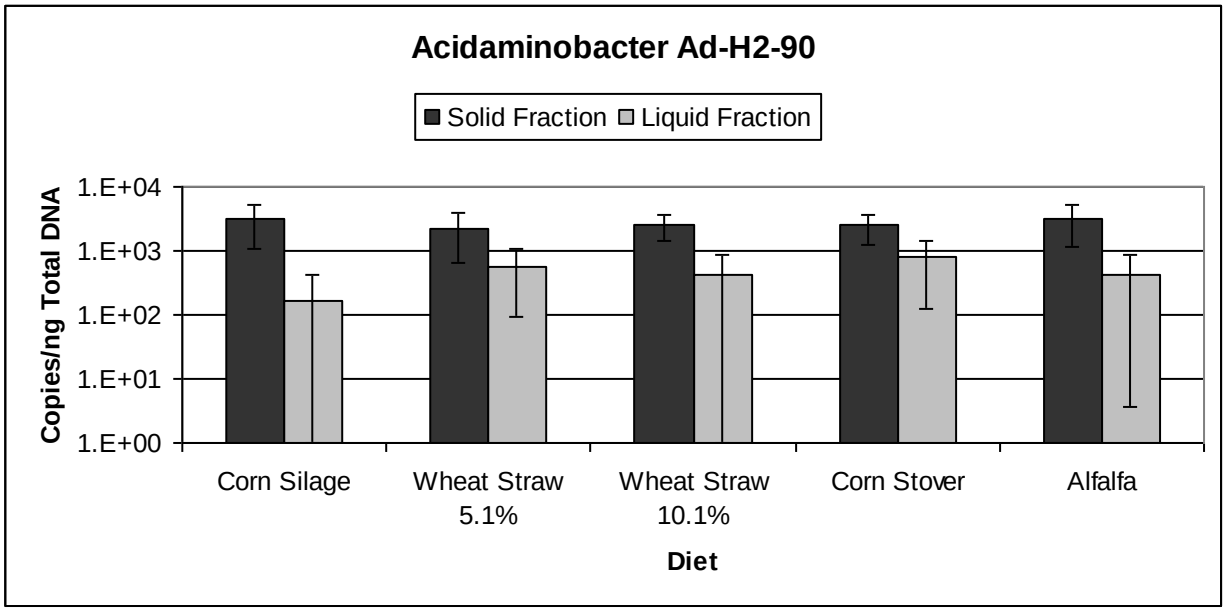

Figure 9. Populations of *Acidaminobacter* sp. Ad-H2-90, an uncultured bacterium recovered from the adhering fraction of rumen content, within the solid and liquid fractions for each dietary treatment.

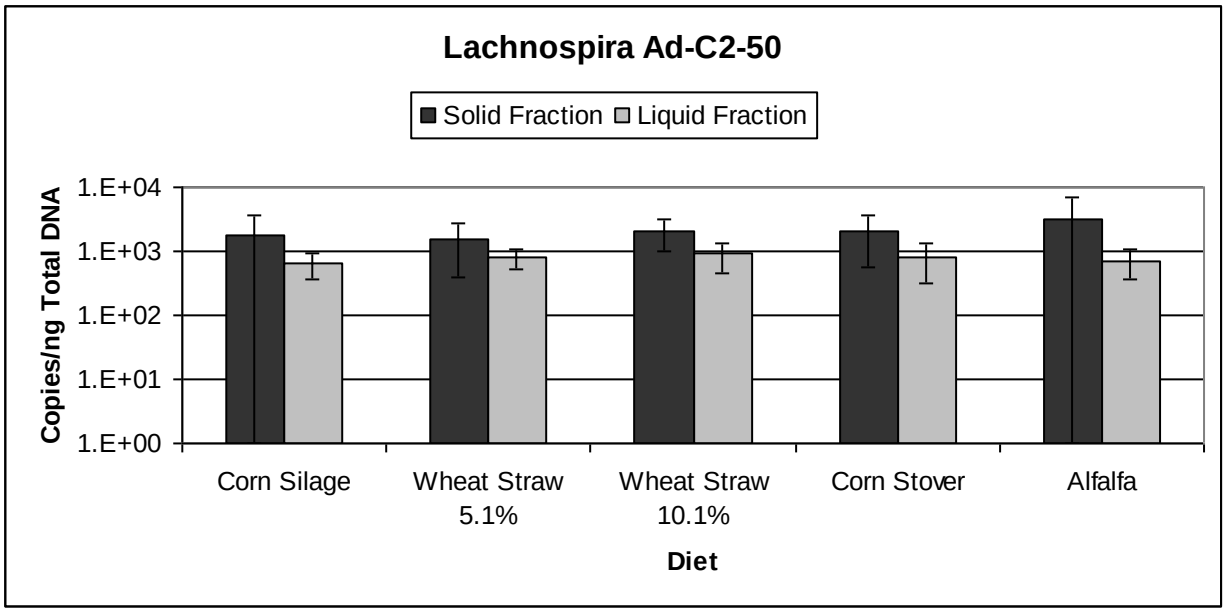

Figure 10. Populations of *Lachnospira* sp. Ad-C2-50, an uncultured bacterium recovered from the adhering fraction of rumen content, within the solid and liquid fractions for each dietary treatment.

Cluster Analysis of DGGE

DGGE banding patterns were very similar between samples for both archaea and total bacteria DGGE profiles. Dendograms of both total bacteria (Figure 11) and archaea (Figure 12) did not show any grouping according to diet, supporting that there was not much difference in bacterial populations between treatments as shown by the real-time PCR data. Samples did tend to cluster together by fraction, indicating that there were differences between the species present in the liquid and solid fractions.

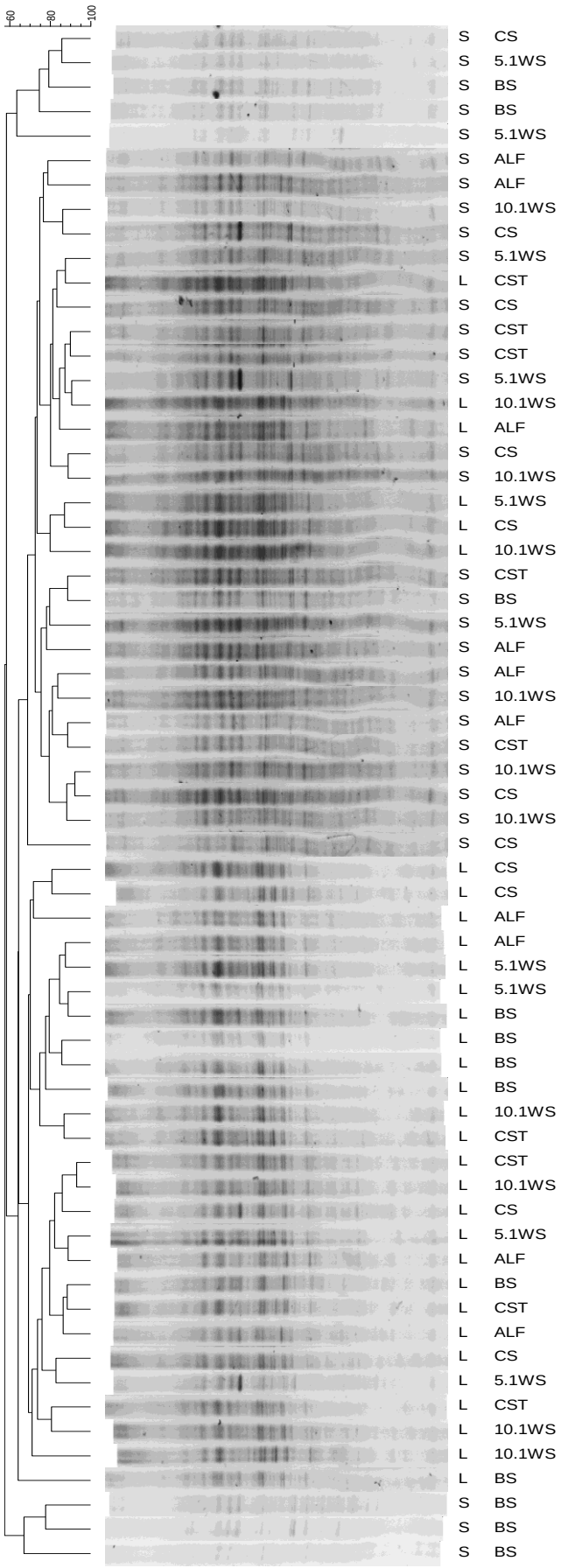

Figure 11. DGGE profiles of total bacteria derived from solid (S) and liquid (L) fractions. BS = Baseline, CS = Corn Silage, CST = Corn Stover, 10.1WS = 10.1% Wheat Straw, 5.1WS = 5.1% Wheat Straw, ALF = Alfalfa.

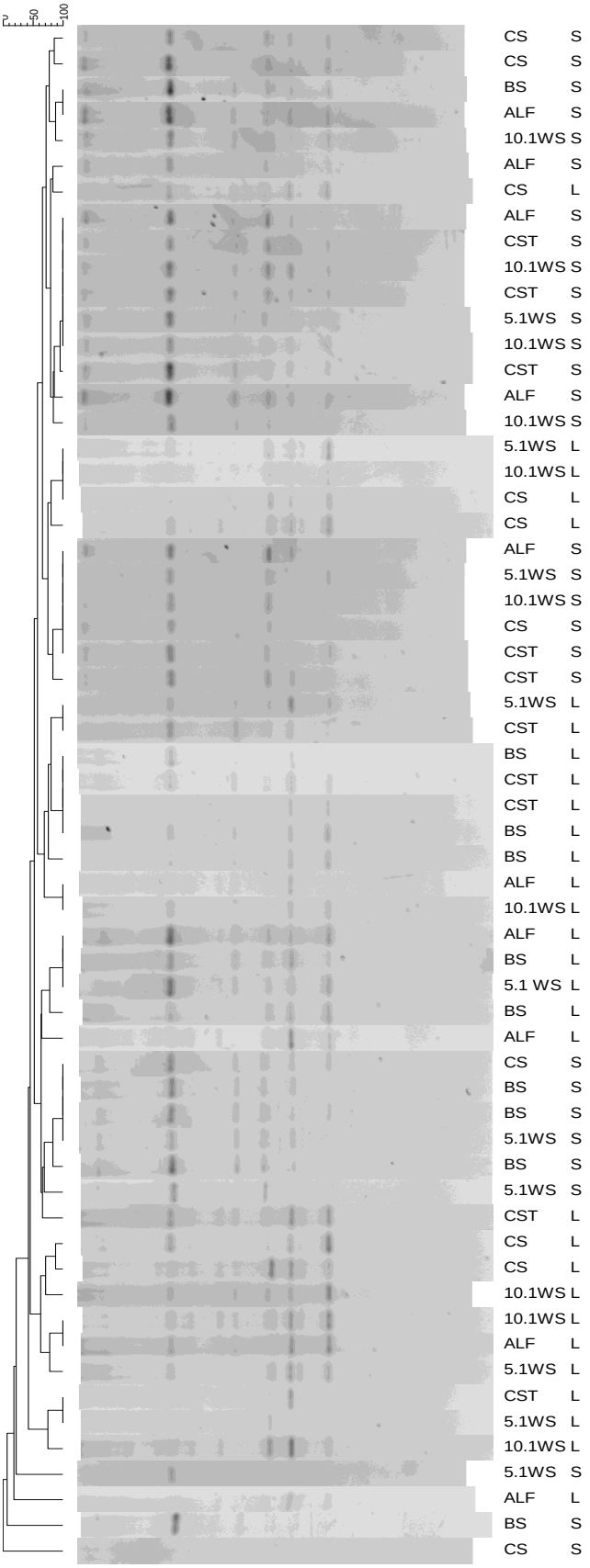

Figure 12. DGGE profiles of archaea derived from solid (S) and liquid (L) fractions. BS = Baseline, CS = Corn Silage, CST = Corn Stover, 10.1WS = 10.1% Wheat Straw, 5.1WS = 5.1% Wheat Straw, ALF = Alfalfa.

Principal Component Analysis

Principal Component Analysis was performed for both the total bacterial and archaeal DGGE profiles. The total bacterial profiles had a PC1 variance of 17.829% and a PC2 variance of 10.047%. On the scatterplot (Figure 13), a few treatment groups tended to cluster together, but only to a limited extent. The solid fractions for the 10.1% wheat straw diet tended to group together on the PC2 axis. The liquid fractions of the baseline (taken before the beginning of the feeding trial) and corn silage treatments also clustered loosely together on the PC1 and PC2 axes.

The archaeal profiles had a PC1 variance of 21.346% and a PC2 variance of 17.674% (Figure 14). While samples tended to overlap, they did not cluster with respect to treatments or fractions, further supporting that there was no obvious relationship between the diet treatments and archaeal populations in the rumen.

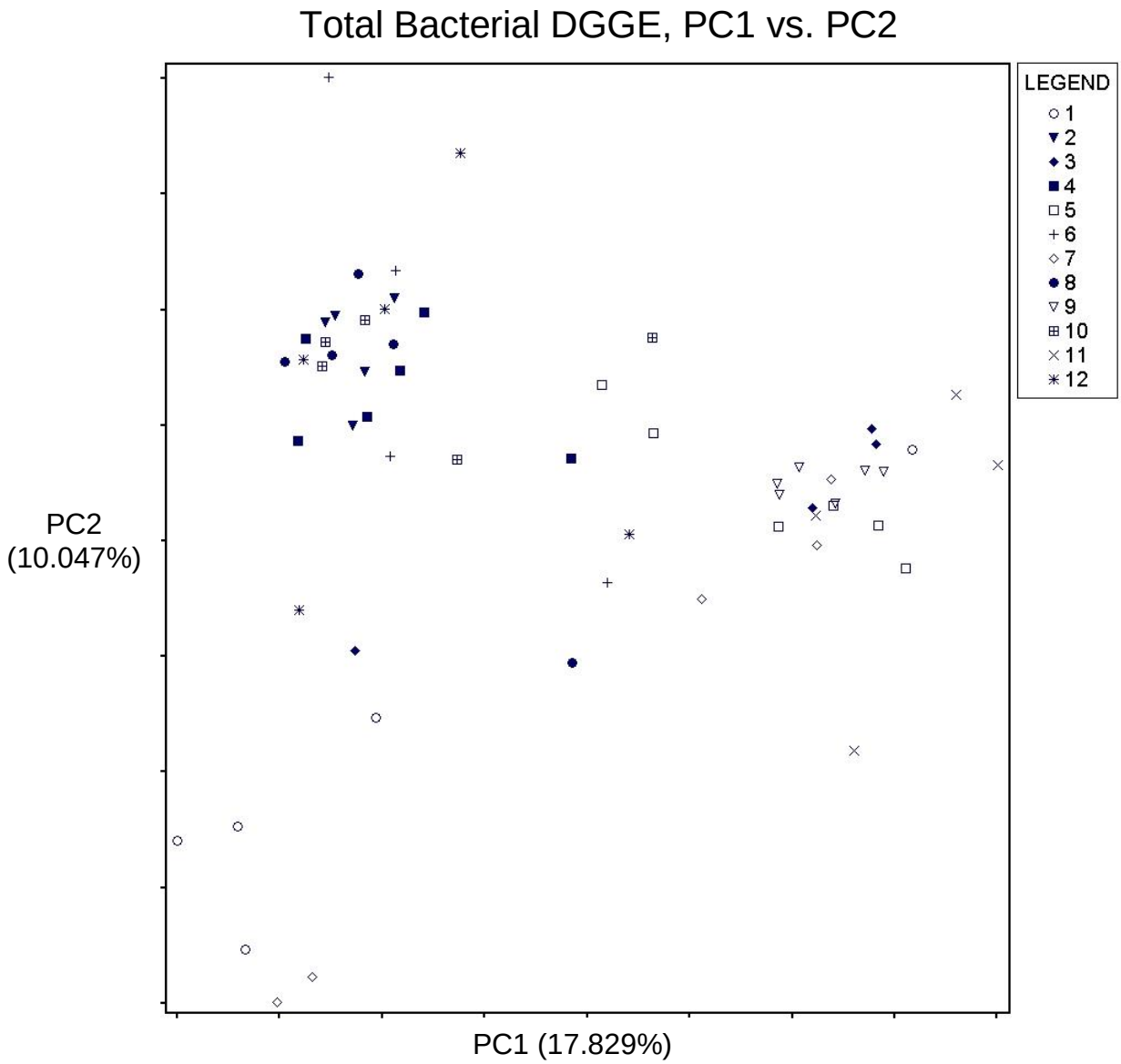

Figure 13. PCA scatterplot of total bacterial DGGE profiles. 1= baseline (collected before the feeding trial began), solid fraction; 2 = baseline, liquid fraction; 3 = corn silage, solid fraction; 4 = corn silage, liquid fraction; 5 = corn stover, solid fraction; 6 = corn stover, liquid fraction; 7 = 5.1% wheat straw, solid fraction; 8 = 5.1% wheat straw, liquid fraction; 9 = 10.1% wheat straw, solid fraction; 10 = 10.1% wheat straw, liquid fraction; 11 = alfalfa, solid fraction; 12 = alfalfa, liquid fraction.

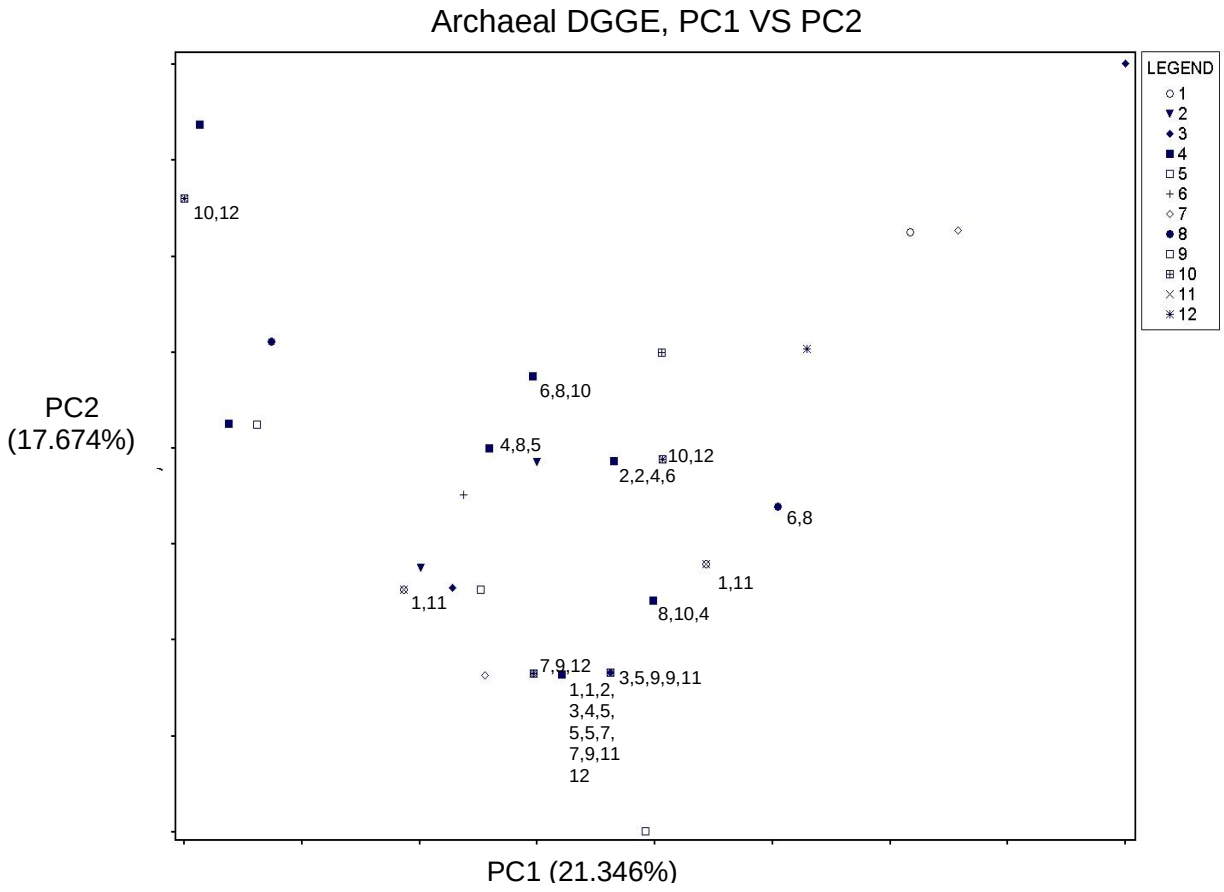

Figure 14. PCA scatterplot of archaeal DGGE profiles. 1= baseline (collected before the feeding trial began), solid fraction; 2 = baseline, liquid fraction; 3 = corn silage, solid fraction; 4 = corn silage, liquid fraction; 5 = corn stover, solid fraction; 6 = corn stover, liquid fraction; 7 = 5.1% wheat straw, solid fraction; 8 = 5.1% wheat straw, liquid fraction; 9 = 10.1% wheat straw, solid fraction; 10 = 10.1% wheat straw, liquid fraction; 11 = alfalfa, solid fraction; 12 = alfalfa, liquid fraction.

Discussion

Samples were separated into solid and liquid fractions in order to assess potential differences between the two fractions and gain a better understanding of each rumen ecosystem. Olubokbukun (1990) found that species present in the rumen fluid, attached to feed particles, and

associated with the feed particles differed, with the majority of the bacteria either associated or attached to the feed particles. Larue et al. (2005) also observed that species varied considerably between the adherent, associated, and liquid fractions in the same animal. These findings are supported by the present study, in which differences in real-time PCR data were observed when comparing the liquid and solid fractions for *Butyrivibrio, Ruminococcus flavefaciens,* and *Acidaminobacter* sp. Ad-H2-90. Cluster analysis of DGGE profiles of total bacteria and archaea were able to group solid fractions and liquid fractions together, further indicating that the combinations of species present were different between the two fractions.

The three novel bacteria species, *Acetivibria* sp. Ad-H1-14, *Acidaminobacter* sp. Ad-H2-90, and *Lachnospira* sp. Ad-C2-50, were analyzed in the hopes of gleaning some information on relatively newly discovered species that have not been cultured and are poorly understood. Traditionally, rumen and other microbial ecosystems were studied based on the species that could be cultured (Rondon et al., 2000; Hugenholtz et al., 1997). However, it is estimated that less than 1% of microorganisms can be cultured in a laboratory setting (Huggenholtz et al., 1997), and Krause et al. (2003) states that the major fibrolytic species may not have been cultured yet. Stevenson and Weimer (2007) found that the most commonly cultured rumen bacteria made up a very small portion of the total bacteria present: *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* each made up 0.5%-1.0% of the total bacteria, while *Butyrivibrio fibrosolvens* and *Ruminococcus albu*s made up less than 0.03%. The present study supported these findings, as most of the individual species made up less than 1%; *R. flavefaciens* was the exception, forming 1.2% of total bacteria in the solid fractions (Table 3). DGGE analysis of total bacteria was used to overcome the typical bias towards culturable species; since DGGE analyzes DNA of both

cultured and uncultured bacteria species, it gives an overall picture of the species present in the rumen.

Table 3. Bacterial species as a percentage of total bacteria within the liquid and solid fraction assessed across dietary treatments.

	Butyrivibrio	*R. albus*	*R. flavefaciens*	*F. succinogenes*	*A.* sp. Ad-H1-14	*A.* sp. Ad-H2-90	*L.* sp. Ad-C2-50
Liquid Fraction	0.413%	0.542%	0.296%	0.106%	0.234%	0.079%	0.198%
Solid Fraction	0.660%	0.701%	1.217%	0.073%	0.459%	0.386%	0.284%

No differences in bacterial population concentrations were observed between dietary treatment groups. This could be explained by the similarities between the diets themselves, which never differed by more than 12% in concentrations of the tested forages. Previous studies analyzed degradation of forage in pure culture, and cultures were offered one substrate exclusively (Matulova et al., 2008, Dehority and Scott, 1967). In the present study, dietary treatment (forage type) had little effect on methanogens, a finding similar to a report by Leedle and Greening (1988) who found that the level of forage in a diet had no effect on methanogen populations. If the amount of forage has little effect on methanogens, then perhaps the type of forage is also inconsequential.

Dry matter intake (DMI), milk yield, milk composition, rumen pH, and concentration of volatile fatty acids were also analyzed for each animal in the present study (Starkey 2009). DMI was lowest for the 10% wheat straw diet, presumably because of gut fill. Body weight, body condition score, milk yield, milk fat, milk protein, and MUN were similar across all treatments. Rumen characteristics were also uniform: rumen pH, concentrations of acetate, propionate,

butyrate, valerate, and isobutyrate and the ratio of acetate to propionate were very similar across treatments ($P > 0.05$). The only difference found was the concentration of isovalerate, which was greatest in the 10% wheat straw diet ($P < 0.05$) (Starkey, 2009).

A Latin square design was utilized to reduce the impacts of both the individual animals' rumen species (Weimer et al., 1999) and differences in feeding periods. The period differences that were observed could be attributed to differences between animals, since the same diet was fed to different animals in each treatment.

Conclusion

There was no difference in bacterial and archaeal populations across the five dietary treatments. This could be a result of the similarity of the five diets, and diets with a greater amount of variation could potentially affect bacterial populations. The lack of differences in archaeal populations is consistent with current literature, indicating that forage type does not affect methanogen activity in the rumen. Differences in microbial species and population sizes were observed between liquid and solid fractions of rumen contents, supporting current literature which states that the adherent and planktonic bacterial populations differ within the rumen.

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